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EXHIBIT C

Quantitative Measurement of Ovalbumin Messenger Ribonucleic Acid Activity

LOCALIZATION IN POLYSOMES, INDUCTION BY ESTROGEN, AND EFFECT OF ACTINOMYCIN D*

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SUMMARY

Procedures are described for the quantitative assay of ovalbumin mRNA in a rabbit reticulocyte lysate protein-synthesizing system. A technique for rapid and efficient separation of labeled immunoprecipitated ovalbumin from nonspecific radioactivity is presented. The capacity of the lysate system for total *de novo* protein synthesis, determined with the use of aurintricarboxylic acid, was found to be correlated with the capacity for ovalbumin mRNA translation. Hemin, a compound that stimulates hemoglobin synthesis in reticulocyte lysates, also stimulates ovalbumin synthesis.

The yield of ovalbumin mRNA activity was used as a criterion in choosing optimal conditions for the isolation of undegraded nucleic acid from oviduct. Phenol extraction of total oviduct homogenate yielded only one-third as much activity as isolation by sodium dodecyl sulfate sucrose gradient. When high concentrations of heparin were used to inhibit ribonuclease, the degradation rate of ovalbumin mRNA activity after homogenization could be slowed enough (half-life = 6.4 hours) to permit nearly quantitative recovery of activity in subcellular fractions. No translation of mRNA fragments could be detected in partially degraded nucleic acid preparations.

Seventy-one per cent of the total ovalbumin mRNA activity sedimented with polysomes with only 5% in the 27,000 $\times$ *g* pellet. Administration of estrogen to immature chicks induced ovalbumin mRNA activity, and continued hormone treatment was necessary for the maintenance of activity. *In vivo* administration of actinomycin D to chicks led to a 65% increase in the rate of ovalbumin synthesis in culture with no change in total or polysomal mRNA levels, providing direct evidence that this drug increases the rate of ovalbumin mRNA translation.

Since the original studies showing that hemoglobin mRNA could be detected and partially purified (1–5), a variety of other eukaryotic mRNA species have been described (6–12). Several features of ovalbumin synthesis in the chick oviduct make the study of ovalbumin mRNA particularly interesting. First, ovalbumin is a tissue-specific product of a well defined cell type, the tubular gland cell (13). Its synthesis represents one of the final events in the cytodifferentiation of primitive epithelial cells to gland cells. Second, ovalbumin synthesis is under hormonal control. Even after formation of tubular gland cells, the presence of estrogen is required for maintenance of ovalbumin synthesis (14). Third, oviduct tissue is available in large quantities, and ovalbumin accounts for more than half of the protein synthesized in the fully differentiated tissue (15). Earlier studies have shown that it is possible to assay ovalbumin mRNA by translation in a heterologous cell-free system (8, 9), identify and isolate ovalbumin-synthesizing polysomes by immunological techniques (15, 16), and partially purify ovalbumin mRNA (17).

Several difficulties are encountered in the quantitative assay of ovalbumin mRNA activity with the reticulocyte lysate cell-free system. These include obtaining adequate sensitivity for the assay of unpurified nucleic acid fractions, obtaining a linear response to added mRNA, and extracting total mRNA from the tissue in undegraded form. In the work presented here we describe methods for the quantitation of ovalbumin mRNA. These have enabled us to answer several questions concerning the subcellular localization of ovalbumin mRNA activity, changes in mRNA levels following estrogen administration and withdrawal, and the mechanism by which actinomycin D preferentially enhances ovalbumin synthesis in the oviduct.

MATERIALS AND METHODS

Chemicals—Estradiol 17β-benzoate was a gift from Schering Corp. Actinomycin D was kindly provided by Merck Sharp and Dohme Research Laboratories. Aurintricarboxylic acid, phosphocreatine, and creatine phosphokinase were products of Sigma Chemical Co. Hemin was purchased from Eastman Kodak Co., and ribonuclease A, from Worthington Biochemical Corp. Glass-distilled water was used for the preparation of all solutions.

Treatment of Animals—Chicks were obtained and treated as previously described (14). White New Zealand rabbits, 2 to 3 kg, were used for the production of antibody and reticulocytes.

Assay of Ovalbumin mRNA Activity—The rabbit reticulocyte lysate was prepared as described earlier (8). The protein-

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synthesizing system used was slightly modified from that of Maxwell *et al.* (18) and differed in the following respects: magnesium acetate was used instead of magnesium chloride; 25 μCi per ml of reaction volume of [4,5-$^3H_2$]leucine (Schwarz-Mann, 40 Ci per mmole) was used instead of [^{14}C]leucine; the nonradioactive leucine concentration (excluding endogenous leucine, see "Results") was 5 μM; the amino acid mixture used was that of Lingrel and Borsook (19) at one-tenth the final concentration described, with the exceptions that leucine and hydroxyproline were omitted, and the final concentration of isoleucine was 46 μM. Reactions were carried out in 250 μl final volume and contained 100 μl of lysate and 75 μl of a mixture of the supplemental components. Incubation was for 1 hour at 25°. After the addition of 30 μl of a solution containing 0.5 mg per ml of ovalbumin and 0.1 M leucine, the reaction mixtures were transferred to Beckman model 152 microfuge tubes and centrifuged at 15,000 $\times$ g_{max} in either the microfuge (1 min) or the Sorvall HB-4 rotor[1] (5 min; a specially made adaptor is required to spin microfuge tubes in this swinging bucket rotor).

Immunoprecipitation of labeled ovalbumin was performed in duplicate by addition of 100 μl of centrifuged lysate reaction mixture to 100 μl of a solution containing sufficient anti-ovalbumin (20) to quantitatively precipitate 5 μg of ovalbumin, 30 μl of a 10% Triton X-100-10% sodium deoxycholate solution, and phosphate-buffered saline (10 mM sodium phosphate, 150 mM NaCl, pH 7.5). After 30 min at room temperature, each reaction mixture was layered on 200 μl of a solution, previously centrifuged at 15,000 $\times$ g,[1] of 1 M sucrose, 0.01 M leucine, 1% Triton X-100, and 1% sodium deoxycholate in a microfuge tube and centrifuged for 5 min at 15,000 $\times$ g_{max} (Sorvall HB-4 rotor). The contents of each microfuge tube were frozen by brief immersion in Dry Ice-acetone, and the tip containing the antibody precipitate was cut off, placed in a scintillation vial, and shaken with 0.7 ml of NCS (Amersham-Searle) at 37° for several hours. Ten milliliters of toluene scintillation fluid (21) were then added, and radioactivity was determined by liquid scintillation spectrometry (efficiency 34%). One unit of ovalbumin mRNA activity is defined as 1 pmole of leucine incorporated into ovalbumin in the lysate system.

Total reticulocyte protein synthesis was determined by spotting duplicate 10-μl aliquots of the centrifuged lysate reaction mixture on Whatman No. 540 filter discs and washing the discs with trichloroacetic acid as described by Bollum (22). The protein was finally dissolved in 1 ml of NCS and radioactivity determined as with antibody precipitate.

Subcellular Fractionation and Preparation of Nucleic Acid—All solutions and glassware used in the preparation of nucleic acid were sterilized by autoclaving for 20 min. Sterile, disposable pipettes and culture tubes were used whenever possible. Oviduct, either fresh or previously frozen in liquid nitrogen, was homogenized at 0° in 8 volumes of polysome buffer (25 mM Tris-HCl, 25 mM NaCl, 5 mM $MgCl_2$, pH 7.5, at 4°) containing 5% sucrose and 500 μg per ml of sodium heparin in a Dounce homogenizer with five to eight strokes of the loose pestle. One volume of 10% Triton X-100-10% sodium deoxycholate was added, and the tissue was further homogenized with three strokes of the tight pestle. For the preparation of total nucleic acid, an aliquot of the homogenate was added to an equal volume of buffer at room temperature containing 2% SDS,[2] 10 mM EDTA, 40 mM sodium acetate, 80 mM Tris-HCl, pH 7.0, at 20° (two times concentrated SDS buffer). In some experiments the homogenate was filtered through several layers of cheesecloth to facilitate pipetting. The mixture was then homogenized at room temperature in a Sorvall Omni-Mixer for 30 s at top speed and centrifuged at 27,000 $\times$ g_{max} for 5 min. One milliliter of 27,000 $\times$ g supernatant fluid was layered over a 10.4-ml, 5 to 20% continuous sucrose gradient in SDS buffer with a 1.0-ml cushion of 40% sucrose in SDS buffer. Nucleic acid was separated from protein, heparin, and other low molecular weight substances by centrifugation at 40,000 rpm in the Spinco SW 41 rotor at 20° for 6 hours. Gradients were collected from the bottom, and A_{260} was monitored continuously with a Gilford spectrophotometer and flow cell. The tubing and flow cell were previously exposed briefly to acid-dichromate-cleaning solution or 0.1% diethylpyrocarbonate to destroy ribonuclease. All nucleic acid sedimenting faster than the midpoint of the trough between 18 S rRNA and supernatant protein was collected, made 0.2 M in NaCl, and precipitated by the addition of 2 volumes of cold (−20°) ethanol. After standing overnight at −20°, the nucleic acid precipitate was collected by centrifugation for 20 min at approximately 3,000 $\times$ g (International model 2), redissolved in 5 mM Tris-HCl, pH 7.5, made 0.2 M in NaCl, and reprecipitated with ethanol. The precipitate was collected by centrifugation, and all traces of ethanol were removed by lyophilization. Immediately before assay in the reticulocyte lysate system nucleic acid fractions were dissolved in water.

A 27,000 $\times$ g supernatant fraction was obtained from the homogenate by centrifugation for 5 min, and nucleic acid was prepared by dilution of an aliquot with an equal volume of SDS buffer (two times concentrated) and processing as described for total nucleic acid. The 27,000 $\times$ g pellet was suspended in SDS buffer and nucleic acid prepared in the same manner.

For the isolation of polysomes, an aliquot of the 27,000 $\times$ g supernatant fraction was diluted with an equal volume of polysome buffer containing 500 μg per ml of sodium heparin. Two milliliters of this solution were layered on a 10.4-ml, 5 to 20% continuous sucrose gradient in polysome buffer containing 500 μg per ml of sodium heparin. Centrifugation for 2 hours at 40,000 rpm in the Spinco SW 41 rotor at 4° was sufficient to pellet polysomes but not monosomes and slower sedimenting substances as judged by optical density profiles. The pellet was suspended in SDS buffer with a glass rod and RNA prepared as described for total nucleic acid.

Measurement of Protein Synthesis in Culture—Synthesis of ovalbumin and total protein in oviduct fragments was measured by the incorporation of labeled amino acids in culture as previously described (20). Labeled ovalbumin was separated from nonspecific radioactivity in the same manner as for the reticulocyte system (see "Assay of Ovalbumin mRNA Activity"). Acid-soluble radioactivity was determined by adding aliquots of 75 μl or less of the trichloroacetic acid-soluble fraction to 0.5 ml of NCS and counting as described above. The amino acid concentration of the trichloroacetic acid-soluble fraction was determined by amino acid analysis in duplicate.

DNA and RNA Determination—When nucleic acid was measured in crude tissue homogenates the method of Burton (23) was used for DNA and that of Munro and Fleck (24) for RNA. In purified samples, combined nucleic acid content was estimated spectrophotometrically after dilution in polysome buffer assuming an $A^{1\%}_{260}$ value of 200. The A_{260}:A_{280} ratio was typically 2.1.

[1] This centrifugation was performed to remove debris that carries nonspecific radioactivity into the immunoprecipitate.

[2] The abbreviation used is: SDS, sodium dodecyl sulfate.

RESULTS

Assay of Ovalbumin mRNA Activity—The reticulocyte lysate system of Maxwell *et al.* (18) was found to be nearly twice as active in the incorporation of amino acids as that used previously for the translation of ovalbumin mRNA (8). The addition of hemin stimulates endogenous protein synthesis in this system to varying degrees depending on temperature of incubation and concentration of hemin (25, 26), apparently by preventing the formation of an inhibitor of initiation (18, 27–29). Hemin was similarly effective in stimulating the synthesis of ovalbumin under the direction of oviduct nucleic acid at 35°. At a hemin concentration of 26 μM that was optimal for the stimulation of endogenous synthesis (2.5-fold), ovalbumin synthesis was also stimulated optimally (5.3-fold). The difference in the degree of stimulation of ovalbumin and endogenous protein synthesis is consistent with the finding of Zucker and Schulman (25) that hemin affects initiation of protein synthesis primarily; ovalbumin synthesis is solely a result of *de novo* initiation, whereas endogenous synthesis consists of both *de novo* initiation and completion of chains. For routine assay of ovalbumin, however, it was desirable to use conditions that did not require hemin, since the degree of stimulation is sharply dependent on hemin concentration, and the optimal concentration varies from one lysate preparation to another (18). Therefore, based on the finding of Maxwell *et al.* (18), that inhibitor formation is delayed for 2 hours at 25°, this temperature was chosen for routine assay, and no hemin was used.

A major difficulty in the quantitation of mRNA activity using the reticulocyte lysate system is the fact that all preparations of nucleic acid depress the incorporating activity of the system to some extent. This observation appears to be universal for all mRNA preparations tested (4, 7–9, 11, 12), as well as for poly(A)[3] (30), poly(U) (30), double stranded RNA (31), encephalomyocarditis viral RNA (32), and heparin.[4] As a result, increasing concentrations of added nucleic acid frequently produce nonlinear responses if the concentrations are high enough. The most satisfactory solution to this problem is to work at the highest possible specific activity of labeled amino acid so that very low levels of nucleic acid can be assayed, thus perturbing the system as little as possible. In an isotope dilution experiment we determined the endogenous level of leucine to be 14 nmoles per ml of reaction volume. At the maximum specific activity obtainable, where added leucine is considerably below this endogenous level, the total incorporating activity of the system was not diminished, indicating that leucine is not rate-limiting at this concentration. Consequently this specific activity was used routinely for the assay of ovalbumin mRNA.

For some tissues it was necessary to add relatively large amounts of nucleic acid causing considerable depression of incorporating activity. If reduced activity resulted from inhibition of elongation only, ovalbumin and endogenous synthesis would be affected equally and one could correct for inhibition by expressing ovalbumin synthesis as a percentage of total protein synthesis. If only initiation were affected, on the other hand, one should express ovalbumin synthesis as a percentage of *de novo* endogenous synthesis. Preliminary experiments indicated that the latter is the case (see also Ref. 32). To estimate the proportion of synthesis resulting from *de novo* initiation, the experiment summarized in Fig. 1 was performed. Increasing amounts of aurintricarboxylic acid were added to the lysate system containing ovalbumin mRNA. This compound specifically inhibits initiation of protein synthesis at low concentrations (below 10^{-4} M) (33, 34). The synthesis of both total protein (initiation plus runoff) and ovalbumin (initiation only) were inhibited, but not to the same extent. Extrapolation of the linear relationship obtained at low inhibitor concentration to zero ovalbumin synthesis indicated that 38% of total protein synthesis resulted from completion of nascent chains in this particular lysate preparation. At higher concentrations, elongation was also inhibited in agreement with earlier studies (33, 34).

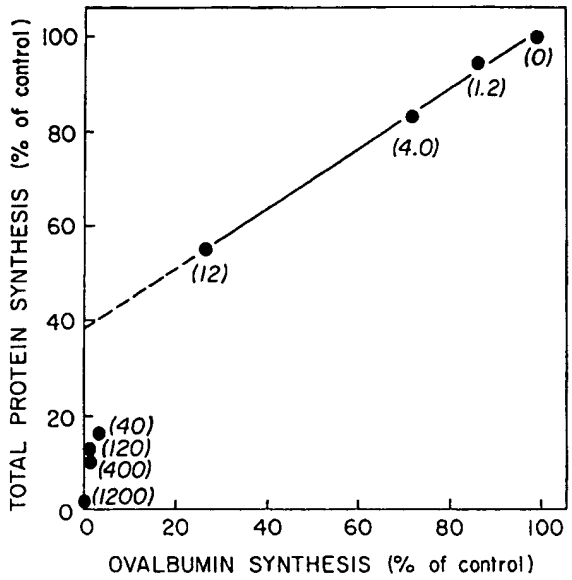

FIG. 1. Estimation of *de novo* protein synthesis in the reticulocyte lysate system. Each assay contained 16 μg of total nucleic acid from chick oviduct (10-day primary estrogen stimulation). Varying amounts of aurintricarboxylic acid were added to the system (*numbers in parentheses* represent the concentration (micromolar) of inhibitor). A linear relationship between total protein synthesis and ovalbumin synthesis, expressed as percentage of controls containing no inhibitor, is obtained at low concentrations of aurintricarboxylic acid. By extrapolating the line to zero synthesis of ovalbumin (*broken line*), one can estimate the proportion of total synthesis which is insensitive to inhibition and, therefore, represents completion of chains.

An example of the application of this information to the quantitation of ovalbumin mRNA activity is given in Table I. It can be seen that the difference between corrected and uncorrected radioactivity is small for the lowest concentration of nucleic acid, but increases for higher concentrations. This was the case in all experiments reported here: uncorrected counts per min were 0 to 32% (average 18%) less than corrected counts per min for the lowest concentration of nucleic acid assayed. Thus the procedure outlined here permits one to average in values obtained with higher, more inhibitory concentrations of nucleic acid without introducing a systematic error. The use of initiating activity as an internal control also facilitates comparison of results between experiments.

Preparation of Nucleic Acid—Our primary concern in developing methods for the isolation of nucleic acid was to obtain a measure of the total ovalbumin mRNA content of a tissue. Two methods commonly used for the separation of nucleic acid from protein, phenol extraction, and treatment with SDS followed by sucrose gradient centrifugation, were compared (Table II). Ovalbumin mRNA activity was tested in total nucleic acid and polysomal RNA fractions prepared by both methods. With the polysome preparations, the yields of mRNA activity were

[3] R. Rhoads, unpublished observations.
[4] M. Summers and D. Sullivan, unpublished observations.

TABLE I

Correction of data for in vitro ovalbumin synthesis based on amount of de novo total protein synthesis

Three concentrations of total nucleic acid from 10-day primary stimulated oviduct were assayed in the standard lysate system. *De novo* total protein synthesis was estimated by subtracting 38% of the total protein synthesis in the control assay, which is the amount due to completion of chains (Fig. 1), from each value for total protein synthesis obtained with different nucleic acid concentrations. Expressed as a fraction of the control, this represents the initiation activity of the system in the presence of that particular nucleic acid fraction. Ovalbumin synthesis data are then calculated as radioactivity per initiation activity (last two columns). A background of 96 cpm, representing nonspecific radioactivity in antibody precipitates from reactions containing no oviduct nucleic acid, has been subtracted from the values in the second column.

Nucleic acid assayed	Ovalbumin synthesis[a]		Total protein[b] synthesis	*De novo* total protein synthesis		Corrected ovalbumin synthesis	
				Radioactivity	Fraction of control		
μg	*total cpm*	*cpm/μg nucleic acid*	*cpm* × 10^{-3}	*cpm* × 10^{-3}		*total cpm*	*cpm/μg nucleic acid*
0	0	0	59.0	37.6	1.00	0	0
15.9	721	45.3	56.5	35.1	0.933	733	48.6
31.8	1240	39.0	52.5	31.1	0.829	1500	47.1
52.8	1530	29.0	45.5	24.1	0.569	2690	50.9

[a] Anti-ovalbumin-precipitable radioactivity in 100 μl of reaction mixture.

[b] Trichloroacetic acid-precipitable radioactivity in 10 μl of reaction mixture.

TABLE II

Comparison of ovalbumin mRNA activity in nucleic acid fractions prepared by different methods

Total nucleic acid was obtained from hen oviduct by SDS-phenol extraction as described earlier (8) except that an Omni-Mixer was used for homogenization. For the preparation of monosomes and polysomes, oviduct was homogenized and centrifuged as described under "Materials and Methods" except that the heparin concentration was 100 μg per ml. One milliliter of the 27,000 × *g* supernatant was layered on a 0.5 to 1.5 M continuous sucrose gradient with a 1.0-ml cushion of 2.0 M sucrose. After centrifugation for 1.6 hours at 41,000 rpm in the SW 41 rotor at 4° the gradient was collected in two fractions: the heavier material consisting of polysomes, and the lighter material consisting of monosomes, subunits, and supernatant protein. These were extracted with phenol as described for total nucleic acid. Pelleted polysomes were prepared by layering 7 ml of the 27,000 × *g* supernatant on 4 ml of 1.0 M sucrose in polysome buffer with 100 μg per ml of heparin and centrifuging as described for gradient polysomes. The pellet was dissolved in phenol buffer (8) and extracted similarly. After ethanol precipitation all phenol-extracted fractions were centrifuged through SDS-sucrose gradients to remove heparin. Total nucleic acid was prepared by the SDS method essentially as described under "Materials and Methods" except that tissue was homogenized directly in SDS buffer in an Omni-Mixer. Polysomes were pelleted as detailed above, dissolved in SDS buffer, and processed as with total nucleic acid. Approximately 30, 60, and 90 μg of each nucleic acid type were assayed.

Methods of nucleic acid preparation and fraction	Yield of nucleic acid	Ovalbumin mRNA activity
	mg/g oviduct	*units/g oviduct*
Phenol		
Total	7.50	968
Polysomes-sucrose gradient	3.63	700
Monosomes-sucrose gradient	1.84	21.3
Polysomes-pelleted	3.55	666
Sodium dodecyl sulfate		
Total	10.00	2880
Polysomes-pelleted	3.59	880

about the same, but for total nucleic acid, mRNA activity was nearly 3 times higher when the SDS method was used. Since the nucleic acid fractions were treated identically after phenol extraction and ethanol precipitation, this result indicates that only one-third of the activity present in an SDS-solubilized homogenate is extracted with phenol (ethanol precipitation in our hands appears to be quantitative for the isolation of mRNA activity).

Table II also shows that large polysomes (an average of 12 ribosomes per polysome (15, 21)) can be pelleted and resuspended in SDS buffer without loss of ovalbumin mRNA activity. Only 3% of the activity recovered from a 5 to 20% sucrose gradient was found in the region of monosomes and slower sedimenting substances. But polysomes contained only 30 to 70% of the total activity regardless of the method of preparation. Three possibilities could account for this loss, either singly or in combination: (*a*) mRNA could be degraded during isolation of polysomes, (*b*) a portion of the mRNA could exist in a form that sediments at 27,000 × *g*, and (*c*) polysomes or other mRNA-containing structures might not completely pellet under the conditions used.

The discrepancy between total and polysomal ovalbumin mRNA content was investigated in several experiments summarized in Table III. Experiment 1 shows that the 27,000 × *g* supernatant fraction initially contained considerably more mRNA activity than polysomes, but if it was allowed to stand at 4° for the length of time required to isolate polysomes, activity decreased markedly to a level below that of polysomes. Thus endogenous ribonuclease activity is still present in spite of added heparin.

The heparin concentration was increased from 100 to 500 μg per ml in Experiment 2 of Table III. This produced a 2.5-fold increase in the yield of mRNA activity in polysomes. Omitting heparin or using diethylpyrocarbonate as described by Williamson *et al.* (5) for the isolation of globin mRNA caused almost complete loss of activity. The yield of mRNA was not significantly increased by raising the heparin concentration from 500 to 1000 μg per ml (Experiment 3, Table III).

The stability of mRNA after homogenization with the increased level of heparin was investigated. Fig. 2 shows that the decay of ovalbumin mRNA activity *in vitro* followed first order kinetics with a half-life of 6.4 hours. A similar experiment performed at 100 μg per ml heparin yielded a half-life of 1 hour.

TABLE III

Effect of ribonuclease inhibitors on recovery of ovalbumin mRNA activity

Hen oviduct was fractionated and nucleic acid extracted by the standard procedures ("Materials and Methods") except that polysomes were pelleted as described in Table II. The centrifugation time for pelleting polysomes in Experiments 1 and 2 was 1.6 hours; in Experiment 3, 3.0 hours. In Experiment 1, one aliquot of 27,000 × *g* supernatant was diluted with SDS buffer immediately after preparation. A second aliquot was allowed to stand at 4° for 1.6 hours before addition of SDS buffer. Diethylpyrocarbonate, used in Experiment 2, was diluted with 4 volumes of ethanol before mixing with aqueous solutions. Approximately 10, 20, and 30 μg of each nucleic acid type were assayed.

Nucleic acid fraction	Ribonuclease inhibitor	Yield of nucleic acid	Ovalbumin mRNA activity
		mg/g oviduct	*units/g oviduct*
Experiment 1			
Total	Heparin, 100 μg/ml	9.77	4520
27,000 × *g* supernatant	Heparin, 100 μg/ml	6.25	3400
27,000 × *g* supernatant, 4° for 1.6 hrs	Heparin, 100 μg/ml	6.35	1640
27,000 × *g* pellet	Heparin, 100 μg/ml	2.56	552
Polysomes	Heparin, 100 μg/ml	2.76	1920
Experiment 2			
Polysomes	None	2.49	48
Polysomes	Heparin, 100 μg/ml	2.16	1490
Polysomes	Heparin, 500 μg/ml	2.76	3700
Polysomes	Diethylpyrocarbonate, 0.1%	2.73	55
Experiment 3			
Total	Heparin, 500 μg/ml	9.40	7750
Polysomes	Heparin, 500 μg/ml	4.75	5990
Polysomes	Heparin, 1000 μg/ml	4.85	6150

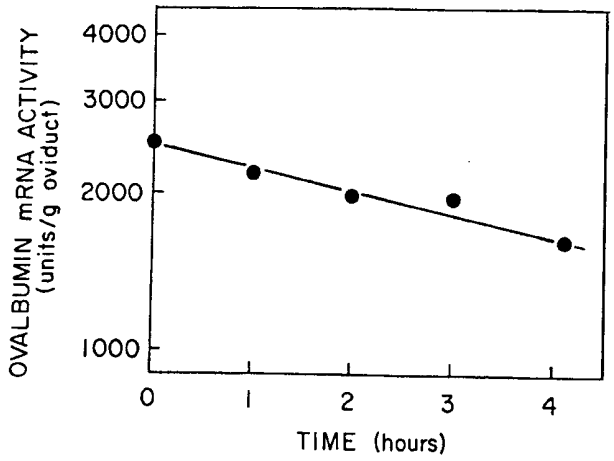

FIG. 2. Time course of ovalbumin mRNA degradation *in vitro*. Oviduct from 10-day primary estrogen-stimulated chick was homogenized as described under "Materials and Methods," and a 27,000 × *g* supernatant fraction was prepared and allowed to stand at 0°. At 1-hour intervals aliquots were transferred to an equal volume of SDS buffer (two times concentrated). Nucleic acid was prepared and ovalbumin mRNA assayed as described. Approximately 5, 10, and 15 μg of each nucleic acid type were assayed.

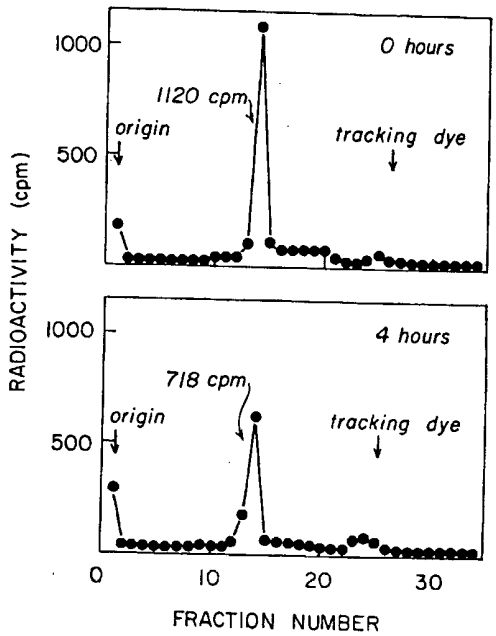

FIG. 3. Electrophoresis of reaction products synthesized by native and "degraded" oviduct nucleic acid. Nucleic acid fractions obtained as described in Fig. 2 were incubated in the reticulocyte lysate system, and antibody precipitates were prepared by the standard method "Materials and Methods". The labeled precipitates were then dissolved and subjected to electrophoresis as described (20). *Upper figure*, nucleic acid prepared from 27,000 × *g* supernatant immediately after centrifugation of homogenate. *Lower figure*, nucleic acid prepared from 27,000 × *g* supernatant after standing 4 hours at 0°.

To verify the specificity of the mRNA assay when partly degraded nucleic acid was used, antibody-precipitable radioactivity from reactions programmed with the same nucleic fractions as described in Fig. 2 were subjected to electrophoresis (Fig. 3). The amount of radioactivity in ovalbumin decreased 36% after a 4-hour incubation of the nucleic acid at 0° in close agreement with 35% seen in Fig. 2. There was, however, no change in the amount of radioactivity that did not migrate in the region of ovalbumin, indicating that the assay measures synthesis of only intact ovalbumin molecules even when programmed with partially degraded nucleic acid.

Subcellular Localization of Ovalbumin mRNA Activity—There is some evidence that mRNA sequences in eukaryotic cells can exist in untranslated forms, both in the nucleus and the cytoplasm (35–39). To approach the problem of whether a major fraction of ovalbumin mRNA exists in untranslated forms,

TABLE IV

Subcellular distribution of ovalbumin mRNA activity in hen oviduct

Fractions of hen oviduct were prepared by the procedures detailed under "Materials and Methods." *Polysomes plus monosomes* in Experiment 1 were obtained by diluting the 27,000 × *g* supernatant with 3 volumes of polysome buffer containing 500 μg per ml of heparin, layering 8 ml over 4 ml of 5% sucrose in polysome buffer and heparin, and centrifuging under the same conditions as for routine polysome isolation. In Experiment 2, oviduct was fractionated without detergents. A 600 × *g* supernatant was obtained by Dounce homogenization, centrifugation, and rehomogenization of the pellet in fresh buffer. After a total of three homogenizations the 600 × *g* supernatant contained 82% of the RNA and 55% of the DNA. Centrifugation at 27,000 × *g* pelleted 70% of the solubilized RNA and 67% of the solubilized DNA. Approximately 3, 6, and 9 μg of each nucleic acid type were assayed.

Homogenizing medium	Subcellular fraction	Yield of nucleic acid	Ovalbumin mRNA activity
		mg/g oviduct	*units/g oviduct*
Experiment 1			
Polysome buffer 1% Triton X-100 1% sodium DOC[a] 500 μg/ml heparin	Total	8.48	6050
	27,000 × *g* supernatant	6.26	5830
	27,000 × *g* pellet	1.66	308
	Polysomes	3.94	4310
	Polysomes plus monosomes	5.52	4450
Experiment 2			
0.25 M sucrose 500 μg/ml heparin	600 × *g* supernatant	3.61	2480
	600 × *g* pellet	1.00	470
	27,000 × *g* supernatant	0.63	236
	27,000 × *g* pellet	2.60	1670

[a] DOC, deoxycholate.

several operational subcellular fractions were prepared from hen oviduct, and ovalbumin mRNA content was assayed (Table IV). In Experiment 1, when detergents were used in the preparation of the homogenate, essentially all of the activity in total nucleic acid was recovered in the 27,000 × *g* supernatant. Only 5% of the activity sedimented under these conditions. The procedure used is not quantitative for the preparation of intact nuclei, but 86% of the total DNA in the homogenate and 9.5% of the RNA were recovered in the 27,000 × *g* pellet. Polysomes contained 74% of the activity in the 27,000 × *g* supernatant fraction. This value was not significantly higher (77%) in a preparation containing monosomes and a portion of the ribosomal subunits as well as polysomes (Table IV, line 5). This supports the earlier conclusion (Table II) that monosomes and lower molecular weight structures contain only a small proportion of the cytoplasmic ovalbumin mRNA activity. To estimate the amount of ovalbumin mRNA activity bound to membrane, fractions of hen oviduct were prepared without the use of detergent (Table IV, Experiment 2). Repeated homogenization was required to release 84% of the ovalbumin mRNA activity into a 600 × *g* supernatant fraction. Nearly all of this activity (88%) sedimented at 27,000 × *g* in contrast with the results of Experiment 1. This suggests that ovalbumin-synthesizing polysomes are attached to very large fragments of endoplasmic reticulum.

Changes in Ovalbumin mRNA Levels during Estrogen Administration in Chicks—Ovalbumin synthesis in immature chick oviducts can be maintained only by continuous administration of

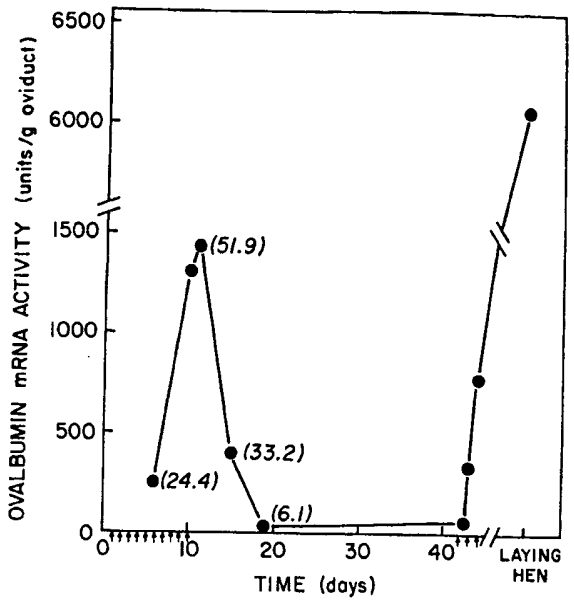

FIG. 4. Ovalbumin mRNA content of chick oviduct tissue during primary estrogen stimulation (1 to 10 days), withdrawal (11 to 41 days), and secondary stimulation (42 to 44 days). Estrogen (1 mg) was administered to chicks as described (14) on days designated with *arrows*. Oviducts were removed and total mRNA activity determined by the procedures discussed under "Materials and Methods" (●). Approximately 9, 18, and 27 μg of each nucleic acid type were assayed. At several points portions of the same tissue used for the preparation of nucleic acid were incubated in culture for 1 hour with tritiated amino acids by methods described (20) in order to determine the relative rate of ovalbumin synthesis. This is expressed as a percentage of total protein synthesis (*numbers in parentheses*).

estrogen (14, 21). Although it seemed likely that ovalbumin mRNA is induced by primary estrogen treatment, it was unclear whether the messenger is degraded after estrogen withdrawal or remains in the cytoplasm in an untranslated form. Fig. 4 summarizes the changes in ovalbumin mRNA in chick oviduct during primary administration of estrogen, withdrawal, and secondary administration. Activity decays soon after cessation of estrogen treatment, indicating that the mRNA is degraded rather than stored. An alternate possibility, although less likely, is that mRNA is converted to an inactive storage form by covalent modification. Secondary administration of estrogen causes a rapid increase in mRNA activity. At several points the relative rate of ovalbumin synthesis expressed as percentage of total protein synthesis was measured in the same tissues used for assaying ovalbumin mRNA content (Fig. 4, *numbers in parentheses*). The two parameters, both of which measure the degree to which oviduct is specialized in making ovalbumin, appear to be generally correlated in immature chicks.

Effects of Actinomycin D on Ovalbumin Synthesis and Ovalbumin mRNA Levels—Techniques for the quantitative extraction and assay of ovalbumin mRNA activity were also employed in an effort to understand the effects of actinomycin D on protein synthesis in the oviduct. Actinomycin D administered concurrently with estrogen during either primary or secondary stimulation completely blocks induction of ovalbumin (20). A paradoxical effect of actinomycin D is noted, however, when the drug is administered to chicks already producing ovalbumin (40). The rate of ovalbumin synthesis actually increases while that of nonsecretory protein remains the same or decreases. Similar increases in the synthesis of a specific protein (or activity) after actinomycin D treatment have been observed in a variety of other

TABLE V

Effect of actinomycin D on protein synthesis and ovalbumin mRNA content

Actinomycin D was administered to seven chicks withdrawn from estrogen for 4 days at a dose of 5 mg per kg of body weight (20). A second group of seven chicks served as controls. After 4.5 hours chicks were killed, the oviducts removed and minced, and duplicate portions of the pooled oviducts placed in culture for 1 hour with tritiated amino acids for the determination of total protein synthesis, ovalbumin synthesis, and amino acid pool specific activity. Specific activities were then used to calculate protein synthesis expressed as nanomoles of amino acid incorporated. Total and polysomal nucleic acid were prepared from the remaining oviduct tissue in triplicate. Variation of ovalbumin mRNA activity within a set of triplicates was strongly correlated with the nucleic acid yield. Consequently the yield of nucleic acid was used as an internal standard to correct the yield of mRNA activity within each group (Columns 4 and 5). This procedure did not affect mean values of mRNA activity. Average per cent S.E.M. before correction: total, 3.7; polysomal, 14.3. After correction: total, 2.1; polysomal, 2.6. Approximately 2, 4, and 6 μg of each nucleic acid type were assayed.

Treatment	Specific activity of amino acid pool	Protein synthesis in culture		Ovalbumin mRNA content of tissue	
		Ovalbumin	Total	Total	Polysomal
	cpm/nmole	*nmoles amino acid incorporated/mg oviduct*		*units/g oviduct*	
Control	227 ± 3[a]	8.02 ± 0.02	24.1 ± 0.1	873 ± 6	598 ± 24
Actinomycin D	317 ± 5	13.2 ± 0.7	31.3 ± 1.1	945 ± 33	574 ± 6

[a] Mean ± S.E.

systems (41–48). In several of these studies the evidence has pointed toward the existence of a cytoplasmic repressor of mRNA translation whose formation is blocked by actinomycin D (41, 48, 49). The implication of models of this type is that increased synthesis of the specific protein results from an increased amount of translatable messenger. An alternate hypothesis, proposed by Palmiter and Schimke (40) to explain this effect in the oviduct system, is that the amount of functional ovalbumin mRNA remains constant, but the rate of its translation increases. Determining the amount of translatable (polysomal) ovalbumin mRNA before and after actinomycin D treatment should, therefore, distinguish between the two proposals.

Table V summarizes an experiment in which the rate of ovalbumin synthesis in culture and the content of ovalbumin mRNA were compared in oviducts from chicks receiving actinomycin D and control chicks. Ovalbumin synthesis increased 65%, but ovalbumin mRNA activity, whether measured in total nucleic acid or in polysomal RNA, remained constant.[5] A further prediction of the cytoplasmic repressor model is that a greater proportion of the specific messenger should be found in polysomes following actinomycin D administration, assuming that in the repressed state the messenger is not in polysomes. Our results, however, show the opposite result, a slight decrease in the proportion of total ovalbumin mRNA in polysomes after treatment. It, therefore, appears that the increase in the absolute rate of ovalbumin synthesis caused by actinomycin D results from an enhanced rate of translation and not an increased amount of functional ovalbumin mRNA.

DISCUSSION

The reticulocyte lysate system is used widely for the detection of mRNA species from a variety of sources (4, 7, 8, 11, 12). Two advantages of such a system over other methods used for this purpose (sedimentation analysis, hybridization, rapid incorporation of radioactivity, base ratio analysis) are that it measures only translatable mRNA molecules, and it can be employed to study the mRNA for a specific protein. As shown in the present study, this system can be used in combination with suitable immunological techniques for rapid and quantitative assay of mRNA activity. Inhibition by nucleic acid fractions can be effectively compensated for when necessary by using the system's estimated capacity for *de novo* protein synthesis as an internal control in each assay. The method described for separation of labeled immunoprecipitates from nonspecific radioactivity is rapid and efficient. Antibody precipitates from reaction containing no oviduct RNA yield only 100 to 200 cpm of nonspecific radioactivity out of 2×10^6 cpm in total radioactivity and 6×10^5 cpm in total protein. The assay is sensitive enough to measure ovalbumin mRNA activity in 8-day withdrawn oviduct which contains only 4.2% as much activity per μg of nucleic acid as 10-day primary stimulated oviduct and 2.2% as much per g, wet weight.

Hemin is being actively studied as a possible regulatory compound in reticulocytes because of its ability to stimulate the synthesis of hemoglobin in the whole cell (50) and in cell-free preparations (25, 26). There is evidence that it prevents the formation of an inhibitor which interacts with initiation factors (18, 27–29). If the hemin effect is in fact specific for hemoglobin synthesis within the reticulocyte, our result that ovalbumin synthesis is also stimulated indicates that hemoglobin initiation factors recognize ovalbumin mRNA. The alternative is that hemin stimulates all protein synthesis in reticulocytes.

The SDS-sucrose gradient method described has several advantages over phenol extraction. Brawerman *et al.* (51) have reported that poly(A) and poly(A)-rich polysomal RNA partition between the phenol and aqueous phases depending on salt and pH conditions. Summers and Sullivan[4] have similarly shown this to occur with ovalbumin mRNA activity. Poly(A) may also be sheared off mRNA at phenol-aqueous interfaces (52). In addition to these problems there is the well documented phenomenon of mRNA aggregation following exposure to phenol (53). This behavior is observed with ovalbumin mRNA activity as well.[6] Some of these effects may account for our finding that only one-third as much activity was recovered in total nucleic acid after phenol extraction at pH 8.0 as with the SDS procedure (Table II). The method described for the isolation of nucleic acid yields reproducible ovalbumin mRNA activity, as shown in Table V, and reproducibility can be further improved by correcting for nucleic acid recovery.

[5] At $p = 0.05$ the change in total mRNA activity following actinomycin D is between −2 and 19%, that for polysomal mRNA, between −7 and −15%, and that for ovalbumin synthesis, between 27 and 102%.

[6] G. S. McKnight and D. Sullivan, unpublished observations.

Although oviduct contains a relatively high level of ribonuclease activity (21), it was possible to slow the degradation rate of mRNA enough with a ribonuclease inhibitor to allow good recovery of activity in polysomes. The level of heparin used routinely in our earlier studies, 100 μg per ml, is sufficient to inhibit completely ribonuclease activity as measured by solubilization of labeled RNA and yields reproducible, high molecular weight polysome preparations (21). It was, therefore, surprising that at 500 μg per ml of heparin the recovery of mRNA activity was 2.5-fold greater (Table III). This illustrates the point that only subtle modifications may be sufficient to inactivate mRNA. This is further seen in the result from Table III, Experiment 2, that 99% of the mRNA activity can be lost with no detectable change in the sedimentation properties of rRNA. (rRNA could, however, be nicked and held intact by secondary structure.)

Studies on subcellular distribution showed that at least on a gross scale ovalbumin mRNA activity is confined to polysomes. This is not unexpected but is contrary to various types of evidence from other systems suggesting that mRNA can exist in nonpolysomal forms (35–39). In light of the discrepancy between the polysomal and 27,000 × *g* supernatant fractions in Table IV a small amount of nonpolysomal ovalbumin mRNA could exist. At least some of the difference, however, must be due to degradation based on the decay rate seen in Fig. 2.

Several conclusions can be drawn from the experiment in Fig. 4. Estrogen is responsible for the appearance of active ovalbumin mRNA. Continued administration of estrogen is required for the maintenance of ovalbumin mRNA activity. Ovalbumin is therefore coded for by an unstable mRNA species, unlike the masked maternal messenger of sea urchin eggs (for review see Ref. 38). Means *et al.* (54) have observed similar estrogen-induced changes in the ovalbumin mRNA activity of a polysomal RNA fraction. The significance of their results is unclear, however, since heparin and detergents were apparently not used in the preparation of polysomes. Fig. 4 also shows that ovalbumin synthesis in culture, expressed as percentage of total protein synthesis, is generally correlated with ovalbumin mRNA content per g of oviduct when measured in immature chicks.

The mechanism of action of actinomycin D on ovalbumin synthesis is clarified somewhat by the experiment in Table V. Messenger RNA cannot be rate-limiting under ordinary circumstances since the rate of ovalbumin synthesis increased 65% with no change in the mRNA level. Palmiter and Schimke (40) have provided independent evidence that actinomycin D causes the rate of peptide chain elongation to increase. The question of which factor is responsible for the increase is, however, still unanswered. It is difficult to extrapolate these results to the other systems that exhibit an actinomycin D effect since the nature and circumstances of the response are different in every case (41–48). In the oviduct system it seems clear that actinomycin D stimulates specific protein synthesis by differentially increasing the rate of mRNA translation, and this hypothesis can be tested in the other systems.

The increase observed in total protein synthesis in culture after actinomycin D was mostly accounted for by ovalbumin synthesis, which is in accordance with the hypothesis (40) that ovalbumin synthesis is stimulated preferentially. The basis for this selectivity is a postulated long half-life of ovalbumin mRNA relative to other cellular messengers. The experiment in Table V confirms that the half-life is much longer than the length of the experiment (4.5 hours), since there was no change in total mRNA activity during this period, but an accurate determination will have to await further experiments.

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